

Serial No.: 09/651,150
Filed: August 30, 2000

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Toso mRNA species of 2.0 (major), 2.8, 3.5 and 4.3 kbp were detected in lymph node and spleen (see Figure 7b). The nucleotide length of the cDNA was 1.9 kbp, suggesting that the additional bands might either be alternative splice products or incompletely processed messages. Toso expression was also observed in peripheral blood leukocytes, thymus (Figure 7b). Expression in bone marrow and fetal liver was much lower than that in lymph node and spleen, as seen after overexposure of the blot (data not shown).—

On page 60, immediately preceding the heading "CLAIMS," please insert the enclosed text entitled "SEQUENCE LISTING."

REMARKS

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached page is captioned "Version with markings to show changes made."

Entry of this amendment is respectfully requested. The specification has been amended to include a Sequence Listing and appropriate reference to the sequences therein and to correct minor typographical errors. The amendments are made in adherence with 37 C.F.R. § 1.821-1.825. This amendment is accompanied by a floppy disk containing the above named sequence, SEQUENCE ID NUMBERS 1-35, in computer readable form, and a paper copy of the sequence information. The computer readable sequence listing was prepared through use of the software program "Patent-In" provided by the PTO. The information contained in the computer readable disk is identical to that of the paper copy. This amendment contains no new matter.

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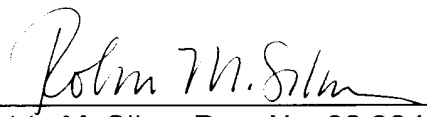
Applicant submits that this amendment, the accompanying computer readable sequence listing, and the paper copy thereof serve to place this application in a condition of adherence to the rules 37 C.F.R. § 1.821-1.825.

Please direct any calls in connection with this application to the undersigned at (415) 781-1989.

Respectfully submitted,

FLEHR HOHBACH TEST
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Dated: 10/22/01


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VERSION WITH MARKINGS TO SHOW CHANGES MADE
IN THE SPECIFICATION

Paragraph beginning at page 5, line 11, has been amended as follows:

– Figure 3 (SEQ ID NOS:3-21) depicts BLAST search results using the Toso gene product. The position of the first amino acid in each sequence is given in the left side of the alignment. Gaps are indicated by dashes. Dark and light shading refer to identical and similar residues, respectively. For sequence alignment of the Toso N-terminus, IgVH (G1HUNM), IgV λ (L1MS4E), TcR V α (RWMSAV), TCR V β (RWHUVY), CD4 (U47924), CD8 chain ~~II~~ II (X04310), Poly Ig R (QRRBG) and immunoglobulin V-set consensus sequence are shown in the alignment. Arrows indicate positions characteristic of many V-set sequences. The sequence of the Toso cytoplasmic domain is aligned with acid sphingomyelinase, insulin receptor substrate 1 (IRS1) and apoptosis inhibitor, IAP, from *Orgyia pseudotsugata* nuclear polyhedrosis virus (~~Op-IAP~~) (Op-IAP).–

Paragraph beginning at page 12, line 4, has been amended as follows:

– Toso proteins may also be identified as being encoded by Toso nucleic acids. Thus, Toso proteins are encoded by nucleic acids that will hybridize to the sequence depicted in Figure 1 (SEQ ID NO:1), or its complement, as outlined herein.–

Paragraph beginning at page 12, line 7, has been amended as follows:

– In a preferred embodiment, when the Toso protein is to be used to generate antibodies, the Toso protein must share at least one epitope or determinant with the full length

protein shown in Figure 2a (SEQ ID NO:2). By "epitope" or "determinant" herein is meant a portion of a protein which will generate and/or bind an antibody. Thus, in most instances, antibodies made to a smaller Toso protein will be able to bind to the full length protein. In a preferred embodiment, the epitope is unique; that is, antibodies generated to a unique epitope show little or no cross-reactivity. In a preferred embodiment, the antibodies are generated to an extracellular portion of the Toso molecule, *i.e.* to all or some of the N-terminal region from amino acid numbers 18-253.—

Paragraph beginning at page 12, line 26, has been amended as follows:

— In the case of the nucleic acid, the overall homology of the nucleic acid sequence is commensurate with amino acid homology but takes into account the degeneracy in the genetic code and codon bias of different organisms. Accordingly, the nucleic acid sequence homology may be either lower or higher than that of the protein sequence. Thus the homology of the nucleic acid sequence as compared to the nucleic acid sequence of Figure 1 (SEQ ID NO:1) is preferably greater than 50 or 60%, more preferably greater than about 70 to 75%, particularly greater than about 80% and most preferably greater than 85%. In some embodiments the homology will be as high as about 90 to 95 or 98%.—

Paragraph beginning at page 13, line 14, has been amended as follows:

— In one embodiment, the nucleic acid homology is determined through hybridization studies. Thus, for example, nucleic acids which hybridize under high stringency to the nucleic acid sequences shown in Figure 1 (SEQ ID NO:1) or its complement is

considered a Toso gene. High stringency conditions are known in the art; see for example Maniatis, *et al.*, Molecular Cloning: A Laboratory Manual, 2d Edition (1989), and Short Protocols in Molecular Biology, ed. Ausubel, *et al.*, both of which are hereby incorporated by reference. An example of such conditions includes hybridization at about 42°C in about 6xSSC with 50% formamide and washing conditions of about 65°C in about 0.2X SSC, 0.1XSDS.—

Paragraph beginning at page 42, line 3, has been amended as follows:

— Jurkat cells (human T cell line) were infected with a retroviral Jurkat T cell cDNA library to screen for cDNAs that encode inhibitory molecules for Fas-induced apoptosis. A retroviral library containing 2×10^6 independent cDNA inserts was constructed from Jurkat cell mRNA by standard methods (Kinoshita and Nolan, unpublished) using a retrovirus vector pBabeMN (Kinoshita, *et al.* (1997)). The library was transfected into an ecotropic virus packaging cell line, ϕ NX-Ampho, as described previously. Jurkat cells were spin-infected with the supernatant from ϕ NX-A cells resulting in 20-40% infection using this method as determined by doping of the library with a marker retrovirus pBabeMN-LacZ or pBabeMN-Lyt-2- α (194 amino acids), which does not have cytoplasmic domain (Tagawa, *et al.*, Proc. Natl. Acad. Sci., **83**:3422-3426 (1986)). Jurkat cells were aliquoted into 96-well plates in media containing 10 ng/ml of anti-human Fas mAb, CH11, (Kamiya Biomedical Company, CA 91359, U.S.A.) for 15 days. Jurkat cells, under conditions empirically derived, were sensitive to Fas-mediated apoptosis with a spontaneous survival rate under our conditions of 2-3

per 10^6 cells. Cells that survived the Fas-mediated killing were identified by outgrowth in the 96 well plate format, expanded, total RNA extracted, and cDNA inserts rescued using RT-PCR (AMV reverse transcriptase from Promega, WI 53711, U.S.A. and Vent DNA polymerase from New England Biolabs, Inc., MA 01915, U.S.A.) with primers 5'-GCT CAC TTACAG GCT CTC TA (LibS; SEQ ID NO:22) and 5'-CAG GTG GGG TCT TTC ATT CC (LibA; SEQ ID NO:23), which were located 282 bp and 56 bp nucleotides upstream and downstream of cDNA insert cloning sites. After an initial denaturation at 94°C for 5 minutes, each cycle of amplification consisted of 30 second denaturation at 94°C, followed by a 30 second-annealing at 58°C and 2 minutes extension at 72°C. After 35 cycles, the final product was extended for 10 minutes at 72°C. The rescued inserts were digested with BamHI-Sall (Promega) or BstXI (Promega), and ligated into the pBabeMN retrovirus vector. The cloned retrovirus containing the novel insert was infected into Jurkat cells. Cells were cultured with 10 ng/ml anti-Fas mAb to confirm whether the inhibitory effect was caused by cDNA inserts of retrovirus. 26 clones were obtained that were resistant to Fas-induced apoptosis, of which 12 carried cDNA inserts. After a second round of anti-Fas screening, one clone, termed here Toso, demonstrated potent inhibition of Fas-induced apoptotic signaling.—

Paragraph beginning at page 43, line 1, has been amended as follows:

- The cDNA insert of Toso was found to contain a 5'-non-coding region of 73 nucleotides, a coding region of 1173 nucleotides (390 amino acids) and a 3'-non-coding

region of 665 nucleotides. (See Figure 1, SEQ ID NO:1). The ATG initiation codon is contained within a standard Kozak consensus sequence. Kyte-Doolittle hydrophathy plot analysis showed that Toso has two hydrophobic regions: the amino-terminal residues from 1 to 17 correspond to the deduced signal sequence (underlined) and residues from 254 to 272 (double underlined) correspond to a presumptive transmembrane region [Hofmann and Stoffel, 1993, analysis was performed using DNAsis-Mac V2.0 (Hitachi Software Engineering, Co. Ltd., Japan)], suggesting that Toso is a type I integral membrane protein. (See Figure 2b). The predicted molecular weight of Toso is 41 kDa. The cytoplasmic region of Toso has a basic amino acid-rich region (from R²⁷⁴ to R³²³), a proline-rich region (from P³³⁴ to P³⁴⁶), and an acidic amino acid-rich region (from E³⁷⁸ to D³⁸⁴) (See Figure 2a and 2b, SEQ ID NO: 2). BLAST search analysis revealed that Toso is a unique gene (Altschul, *et al.*, (1990)). The extracellular domain of Toso has homology to the immunoglobulin variable (IgV) domains, which is characterized by motifs in the β -strand B, D and F regions, (residues VTLTC (SEQ ID NO:24), RV(or F~~I~~ or I) and DSG(or A)-Y-CA (SEQ ID NO:25))(Williams and Barclay, *Ann. Rev. Immunol.*, 6:381-405 (1988)). Importantly, the cysteines in the IgV-like motif VTIKC (SEQ ID NO:26) at position 33 in Toso, as well as the cysteine in the IgV-like motif DSGVYAC (SEQ ID NO:27) at position 98, are appropriately distanced as in other IgV-like domains to form a disulphide bond. Toso also contains within the Ig domains two additional cysteines that are not conserved in other IgV-like domains. Thus, the presumptive extracellular domain has all the

requisite features that demarcate it as a potential IgV-like domain. The cytoplasmic region of Toso has partial homology to FAST kinase, acid sphingomyelinase, insulin receptor substrate-1 (IRS-1) and the apoptosis inhibitor from *Orgyia pseudotsugata* nuclear polyhedrosis virus (~~Op-IAP~~) (Op-IAP) (Figure 3), which might function to initiate some of the signaling systems acted upon by Toso.—

Paragraph beginning at page 43, line 27, has been amended as follows:

— Poly (A)⁺ RNA was prepared from Jurkat cells stimulated for 24 hours with 10 ng/ml PMA (SIGMA) and 500 ng/ml Ionomycin (SIGMA). The first strand of cDNA was synthesized with 10 µg Poly (A)⁺ RNA using oligo-dT primers and performed PCR with primers, 5'-AGA ATT CTC TCT AGG GGC TCT TGG ATG (SEQ ID NO:28; See Figure 1(SEQ ID NO:1) where the EcoRI site is underlined) and 5'-ATA AAG CTT CTC AGG GCA CAG ATA GAT GG(SEQ ID NO:29; HindIII site is underlined), which were located 23 bp and 136 bp nucleotides upstream and downstream of the Toso coding region, respectively. The 1.3 kbp fragment was ligated into pBluescript SK(+). Five independent clones were picked up and sequenced using cycle sequencing ready reaction kit (Perkin Elmer). The deduced amino-acid sequences from the five independent clones were completely identical to the gene from the cDNA library screening, although two silent mutations were found within the original gene as compared to the PCR consensus sequences.—

Paragraph beginning at page 44, line 8, has been amended as follows:

– The Toso gene was mapped to a human chromosome by using a panel of 17 human X Chinese hamster hybrid cell lines derived from several independent fusion experiments (Francke et al., 1986). PCR primers used to amplify Toso sequence derived from the 3' untranslated region were 5'-AGA GGC ATA GCT ATT GTC TCG G (SEQ ID NO:30; sense; located 369 bp downstream of the coding region), and 5'-ACA TTT GGA TCA GGG CAA AG (SEQ ID NO:31; anti-sense; 508 bp downstream of the coding region). The size of the PCR product was 159 bp. The PCR conditions were 94°C, 90 seconds; then 35 cycles of 94°C, 20 seconds; 55°C, 30 seconds; 72°C, 45 seconds; followed by 72°C, 5 minutes. Specific PCR products were obtained from human genomic DNA, and hybrid cell lines that carry human chromosome 1. The PCR product was sequenced to confirm its identity. –

Paragraph beginning at page 48, line 11, has been amended as follows:

– Recently several groups have reported cFLIP is a caspase-8 inhibitor. We performed semi-quantitative RT-PCR to detect cFLIP mRNA expression. To detect cFLIP mRNA expression, a 1.1 kbp fragment (998-2061) of the cFLIP gene (U97074) was amplified with primers 5'-GGG AGA AGT AAA GAA CAA AG (SEQ ID NO:32) and 5'-CGT AGG CAC AAT CAC AGC AT (SEQ ID NO:33) for 35 cycles as described above. The sequence of the 1.1 kbp PCR product was verified using cycle sequencing ready reaction kit (Perkin Elmer, CA 94404, U.S.A.). As a control, β -actin cDNA was amplified for 15 and 25 cycles as described above. cFLIP expression was induced by Toso (Figure 5b). These results strongly suggest that the extracellular domain of

Toso inhibits Fas-induced apoptosis by preventing caspase-8 processing through cFLIP upregulation. –

Paragraph beginning at page 53, line 5, has been amended as follows:

– Poly (A)⁺ RNA was prepared from Jurkat cells or Jurkat cells stimulated for 24 hours with 10 ng/ml of phorbol 12-myristate 13-acetate (PMA; SIGMA Chemical Company, MO 63178, U.S.A.) and 1 µg/ml phytohemagglutinin (PHA; SIGMA) or 10 ng/ml PMA and 500 ng/ml Ionomycin (SIGMA). Poly (A)⁺ RNA (5µg) was subjected to electrophoresis through 1% agarose gel containing 2.2 M formaldehyde, and transferred to Hybond N⁺ membrane (Amersham Life Science Inc., IL 60005, U.S.A.). Hybridization was carried out according to the manufacturer's recommendation. A specific probe for the Toso coding region (1.2 kbp) was synthesized with PCR from pBabeMN-Toso using 5'-AGG GGC TCT TGG ATG GAC (TosoS; SEQ ID NO:34) and 5'-CTG GGG TTG GGG ATA GC (TosoA; SEQ ID NO:35). As a control probe, the human β-actin cDNA control probe (CLONTECH Laboratories, Inc., CA 94303-4230) was used. Probes were labeled with ³²p using a random-primed labeling kit, Prime-a-Gene (Promega). Human RNA Master Blot and Human Immune System Multiple Tissue Northern Blot II (CLONTECH Laboratories) were used to survey Toso mRNA expression in several human tissues. Toso expression was observed in lymph nodes, lung and kidney. In addition to these tissues, we detected faint signals from spleen, thymus, liver, heart and salivary gland. Tissues which were analyzed for Toso mRNA include: A1: Whole brain, A2: Amygdala, A3: Caudate nucleus, A4:

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Cerebellum, A5: Cerebral cortex, A6: Frontal lobe, A7: Hippocampus, A8: Medulla oblongata, B1: Occipital lobe, B2: Putamen, B3: Substantia nigra, B4: Temporal lobe, B5: Thalamus, B6: Subthalamic nucleus, B7: Spinal cord, C1: Heart, C2: Aorta, C3: Skeletal muscle, C4: Colon, C5: Bladder, C6: Uterus, C7: Prostate, C8: Stomach, D1: Testis, D2: Ovary, D3: Pancreas, D4: Pituitary gland, D5: Adrenal gland, D6: Thyroid gland, D7: Salivary gland, D8: Mammary gland, E1: Kidney, E2: Liver, E3: Small intestine, E4: Spleen, E5: Thymus, E6: Peripheral leukocyte, E7: Lymph node, E8: Bone marrow, F1: Appendix, F2: Lung, F3: Trachea, F4: Placenta, G1: Fetal brain, G2: Fetal heart, G3: Fetal kidney, G4: Fetal liver, G5: Fetal spleen, G6: Fetal thymus, G7: Fetal lung. (Figure 7a). Using Human Immune System Multiple Tissue Northern Blot II, and film exposed at -70°C with an intensifying screen for one day, endogenous Toso mRNA species of 2.0 (major), 2.8, 3.5 and 4.3 kbp were detected in lymph node and spleen (see Figure 7b). The nucleotide length of the cDNA was 1.9 kbp, suggesting that the additional bands might either be alternative splice products or incompletely processed messages. Toso expression was also observed in peripheral blood leukocytes, thymus (Figure 7b). Expression in bone marrow and fetal liver was much lower than that in lymph node and spleen, as seen after overexposure of the blot (data not shown).—

On page 60, immediately preceding the heading "CLAIMS," the enclosed text entitled "SEQUENCE LISTING" was inserted into the text